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14. ABSTRACT Environmental recovery and identification technology for Select Agents (potential biological warfare or terrorism agents) requires the capability of a flexible and rapid response, whose rapidity and flexibility exceed the current capability of immunodiagnostic assays. These new assays also need to be brought as far forward in the "Area of Responsibility" as possible to facilitate the appropriate sequestration of exposed areas, handling of exposed individuals and expected casualties. Therefore, any such new assays must be extremely robust and require a minimal logistic tail. We present here examples of such a new technology, Aptamer-Linked Immobilized Sorbent Assays (ALISA). A form of this aptamer-linked assay was used recently in Houston, Texas, to address a tularemia alarm of Biowatch. It compared favorably to a number of standard microbiological techniques and immunoassays. We present here formats for this assay that go further to meet the robustness requirement than the standard ELISA-like format.					
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### **33. SELECT AGENT RECOVERY AND IDENTIFICATION USING APTAMER-LINKED IMMOBILIZED SORBENT ASSAY**

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#### **INTRODUCTION**

Environmental recovery and identification technology for Select Agents (potential biological warfare or terrorism agents) needs to be flexible and rapid. It needs to overcome the current limitations of immunodiagnostic assays (extensive processing of samples, refrigeration of reagents, washing steps and number of reagents required). These new assays also need to be brought as close to the place of collection of samples (whether these be from humans, animals or inanimate sources) as possible to quickly determine the extent of contaminated areas, number and identity of potentially exposed individuals and the number of expected casualties based on the level of exposure. Therefore, any such new assays must be extremely robust and require minimal technical skill and support. We will present an example of such a new technology, Aptamer-Linked Immobilized Sorbent Assay (ALISA). The ELISA-like format has previously been compared to a number of standard assays used in the identification of *Francisella tularensis*. We will present here other formats for this assay that use quantum dots (nanocrystals) and single reagents composed of complexes of aptamers and quantum dots that are quenched until a specific biological agent is introduced into the assay preparation. Because the latter format does not require washing or multiple refrigerated reagents, it goes further to meet the robustness requirement than the standard ELISA-like format.

#### **MATERIALS AND METHODS**

General Design of the Formats. Fig. 1 shows the basic design of the aptamer-linked complex of DNA complementary strands covalently linked to a fluorescent nanocrystal (quantum dot, Evident Technologies, Inc., Troy, New York), and a fluorescent quencher. Optionally, a magnetic particle may be included in the complex. The nanocrystal and quencher are on separate DNA strands. In Fig. 1, one strand of a duplex DNA molecule is linked to the quantum dot by an amide bond. It does not matter if the aptamer or the complementary strand is attached to quantum dot. However, the strand that is not attached contains a non-radiative quencher. Upon addition of the aptamer's target, aptamer and complementary strands are separated by binding of the target to the aptamer. The quencher on the complementary strand is separated from the quantum dot. The nanocrystal fluorescence is de-quenched and observable by eye, a fluorescent reader (fluorometer) as illustrated in Fig 1, or with the aid of a microscope (Fig 3).

Another example comprises a complex of aptamer duplex covalently linked to fluorescent nanocrystal (quantum dot), and a fluorescent quencher, all attached to the bottom of the well of a microtiter plate. In an alternative format, a magnetic particle (micron-sized or a nanoparticle) is used to attach the complex to the bottom of the wells of a plate or microscope slide by a magnet placed under the plate or slide. The magnetic particle (not shown) facilitates the separation of the two strands by magnetic capture of one of them, being attached only to one of them, either covalently by conjugation chemistry, or by a DNA positive and negative strand complementation different from that of the aptamer double strand being separated. This complementation of the magnetic particle DNA may be made covalent by chemically cross-linking the two complementary strands. The de-quenched complex is either magnetically or covalently immobilized on the bottom of the wells of the microtiter plate so that the supernatant can be removed or washed away containing the freed quencher strand of DNA.

Preparation of a Reactive Plate. A maleic anhydride plate (Pierce Biotechnologies, Rockford, Illinois) was reacted with an amino dPEG24 acid (polyethylene glycol linker) to provide a tether to the surface of the plate. This plate was allowed to react overnight in carbonate buffer on the Jitterbug plate shaker. The contents of each well were discarded and washed twice with 200 microliters of PBS pH 7.0

buffer, then once with 200 microliters of methanol. Next, the carboxylic acid end of this tether was then reacted with NHS (N-hydroxy succinimide) and EDC (1-ethyl-3-[3-dimethylamino-propyl]carbodiimide hydrochloride) to couple NHS to the carboxylic group and set it up for reaction with a primary amine. The reaction was carried out in methanol. The plate was washed twice with methanol and the well contents covered in methanol. The plate was covered in parafilm and stored in the refrigerator.

Preparation of Annealed Aptamer Complex. The aptamer strand of 40 nucleotides was annealed to its complement which was 21 nucleotides long. Either the aptamer contained a 5' amine and the complementary a 3' quencher or the aptamer contained a 3' quencher and the complementary strand a 5' amine. Further a 3' amine could be used with a 5' quencher. The strands were annealed in 10 mM NaCl, 0.1 M MOPS (3-(N-morpholino) propanesulfonic acid) buffer, pH 7.2, by heating to 85 C for fifteen minutes in a water bath and while still in the water bath cooled to room temperature. Strands were stored refrigerated.

Annealing the Shiga Toxin Aptamer. Oligonucleotides were purchased from Biosearch technologies, Inc., Novato, California. The Plus STJ-9 sequence was 5' G GTA ACT AGC ATT CAT TTC CCA CAC CCG TCC CGT CCA TAT 3' amine. The Negative STJ-9 was 5' ATA TGG ACG GGA CGG GTG T 3' BHQ2 (Black Hole Quencher 2). The numbers of moles of the two strands were compared. The strand with the largest number of nanomoles was dissolved in 1 ml of 0.1 M MOPS, 10 mM NaCl, pH 7.2, and a volume transferred to the other oligonucleotide such that the Negative STJ-9 strand would be in excess. This was to assure complete annealing of the amino labeled positive strand; it is essential to have no unannealed Plus STJ-9 present after the annealing step. The oligonucleotides were annealed, wrapped in aluminum foil to prevent bleaching of the quencher (BHQ2), with stirring at 75 to 80 C for 15 minutes. The solution was cooled in the water bath to room temperature before refrigeration.

Preparation of Positive Control. Plus STJ-9 was dissolved in 1 ml of MOPS buffer. A volume of plus STJ-9 was mixed with carboxyl functionalized Birch Yellow T1 EviTags such that the ratio of oligonucleotide to EviTag was 4:1. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) dissolved in MOPS buffer was added to the mixture: dissolved 5 mg EDC in 5 ml of 0.1 M MOPS, pH 7.2, added 1 ml to the reaction, and shaken on a rotary stirrer at room temperature. This was repeated approximately every 15 to 20 minutes for a total of five additions, after which the reaction was allowed to proceed at room temperature for another 2 hours. To isolate the EviTags from the reactants and products, an Amicon Ultra Centrifugal Spin Filter was used with a molecular weight cut off of 100,000 Daltons. The EviTags were washed once with 0.05% Tween 20 in PBS and several times with PBS and concentrated to their initial volume before refrigeration.

Quenching of Quantum Dots and Immobilization onto Plate. The annealed strands were conjugated to T1 or T2 Carboxyl Birch Yellow quantum dots (Evident Technologies, Inc., Troy, New York). These dots and strands were mixed with a molar ratio of approximately 8 duplex DNA strands per dot. The reaction was carried out in 0.1M MOPS, pH 7.0, buffer supplemented to a concentration of 10 mM NaCl. Five mg of EDC was dissolved in 5 ml of MOPS buffer, and 1 ml added to the reaction. This was repeated three to five times (usually a total of 5 mg of EDC was added). The dots were allowed to react overnight and EDA (~ 16 mg) was added in the morning along with additional amounts of EDC (~ 3 mg) to couple the EDA. The dots were filtered (Amicon Ultra Spin filters, 100,000 NMCO) and washed with PBS and Tween 20 (0.025%). The dots were washed enough to remove all the unreacted EDA. These dots were then added (50 microliters) to a plate with NHS activated carboxyl groups via the amino dPEG24 acid – tether. The plate was allowed to shake for three hours and then left to stand over night without shaking. The next morning the wells were washed three times with 200 microliters PBS.

Plate Assay. Shiga Toxin, purchased as a lyophilized powder in PBS (Toxin Technologies, Sarasota, Florida), was reconstituted to two milliliters using deionized water. This brought the concentration of toxin to 0.25 mg/ml.

Ovalbumin, purchased from Sigma-Aldrich (Sigma Aldrich, St. Louis, Missouri), was also reconstituted in deionized water to bring the final concentration to 1.0 mg/ml. Increasing milliliter quantities of both Shiga Toxin and ovalbumin were added in triplicates to wells containing immobilized dots. These micro liter quantities ranged from three up to one hundred. The total volume of the wells was brought to 100 microliters using PBS. The plate was allowed to shake on the Jitterbug plate shaker for one hour, starting at 25 C and ramping up to 37 C. The contents of each well were discarded and washed twice with 200 microliters PBS. The wells were then reconstituted in 100 microliters PBS and read using the Synergy Plate Reader.

Preparing the Quenched Quantum Dots for Immobilized Analyte Plate Assay. The annealed oligonucleotide was mixed with carboxyl functionalized Birch Yellow T1 EviTags at a ratio of oligonucleotide to EviTag of approximately 7:1. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) dissolved in MOPS buffer and was added to the mixture: dissolved 5 mg EDC in 5 ml of MOPS and added 1 ml to the reaction and shaken on a rotary stirrer at room temperature. This procedure was repeated approximately every 15 to 20 minutes for a total of five additions, after which the reaction was allowed to proceed at room temperature for another 2 hours. To isolate the EviTags from the reactants and by-products, an Amicon Ultra Centrifugal Spin Filter was used with a molecular weight cut off of 100,000 Daltons. The EviTags were washed several times with PBS and concentrated to their initial volume before refrigeration.

Immobilized Analyte Plate Assay. Shiga Toxin was coupled to Reacti-Bind Maleic Anhydride Plates (Pierce Biotechnologies). Lyophilized Shiga toxin powder (Toxin Technologies) was reconstituted in 2 ml of DI water. Ovalbumin (Sigma Aldrich) was reconstituted in DI water to give a molar concentration similar to that of Shiga toxin. A bicarbonate buffer of pH 9.2 was prepared.

To each well of a 96-well Reacti-Bind Maleic Anhydride Plates (Pierce Biotechnologies) was added 50 microliters of either Shiga toxin or ovalbumin and 50 microliters of bicarbonate buffer. Both proteins were allowed to react with the plate for two hours at room temperature. The contents of each well were discarded, and washed twice with PBS, pH 7.4. Quenched Birch Yellow quantum dots were then added to wells and allowed to react at 37C for thirty minutes and at room temperature for thirty minutes with shaking. The wells were then washed three times with 200 microliter aliquots of PBS and reconstituted in 100 microliters of PBS. The wells were then read by a Synergy Plate reader system. Either Shiga toxin or ovalbumin was immobilized on Pierce Reacti-bind plates. Fifty microliters of protein solution were added to the wells (Shiga toxin conc 0.25 mg/ml, total protein 1.0 mg/ml; ovalbumin 0.5 mg/ml). Number of reactive sites per well are 110 picomoles. Maximum amount of Shiga toxin in a well was approximately 3 micrograms ( $5 \times 10^{-11}$  moles).

## RESULTS AND DISCUSSION

Quantum Dot Dequenching. To each well was added the indicated volume (Fig. 2) of the aptamer-quantum dot complex (concentration of qdots was 4.5 nanomoles/ml). Twenty five microliters contained 0.112 nanomols of qdots. Ratio of aptamer:qdots was 8:1. As the volume of aptamer-quantum dot complex increased, the fluorescence showed a linear increase from the wells with Shiga toxin, indicating the aptamer-quantum dot complex was binding to the toxin and dequenching. The control, ovalbumin, showed no such increase in fluorescence, indicating that the aptamer-quantum dot complex was not binding either specifically or non-specifically to the ovalbumin or the well.

This example does not require extensive washing (at most a one step separation of the freed quencher or complementary metallic nanoparticle strand from the magnetic capture nanoparticle strand) and does not require separate capture and reporter anti-ligands. It is not a typical sandwich assay in which a separate capture anti-ligand (like antibody) and a separate reporter anti-ligand (like fluorescent antibody or enzyme-linked antibody) must be added in separate steps with their accompanying washing steps.

The results of the microtiter-bound aptamer/quenched quantum dots are also displayed in Fig. 2. The control wells to which ovalbumin was added showed no increase in fluorescence across the entire

range of the experiment. However, while the increase in fluorescence of the immobilized aptamer-quantum dot complex was not linear, every well to which Shiga toxin was added showed an increase in fluorescence across the entire range of the experiment (0.10 micrograms to 25 micrograms of Shiga toxin added).

Fig 3 shows the magnetic bead aptamer quantum complex for anthrax spores interacting with spores. The mere one-step interaction with the spores led to increased fluorescence observed microscopically.

## CONCLUSIONS

The examples given here of the aptamer-linked immobilized sorbent assay (ALISA) do not require extensive washing (at most a one step separation of the freed quencher strand from the covalently bound complex or the magnetically bound complex) and do not require separate capture and reporter anti-ligands. This ALISA is not a typical sandwich assay in which a separate capture anti-ligand (like antibody) and a separate reporter anti-ligand (like fluorescent antibody or enzyme-linked antibody) must be added in separate steps with their accompanying washing steps.

The indirect assay measures the interference with the baseline de-quenched fluorescence from adding free complex to the bound control agent. It can measure antibody in the sera of a patient against the agent (when isolating the agent is not practicable) or can be used to measure interference with bound de-quenched fluorescence of the complex when soluble antigen activates quenched complexes that are removed when the supernatant is washed out.

Finally, some other variations of this example are possible. Although the bound complex in microtiter plates may be read in situ for agent or by interference for antibody or competition with control bound agent, it can also be transferred by the release of the magnetically captured agent for further analysis by orthogonal methods such as PCR performed on the DNA, from the magnetically captured agent, or cultured directly off these complexes.

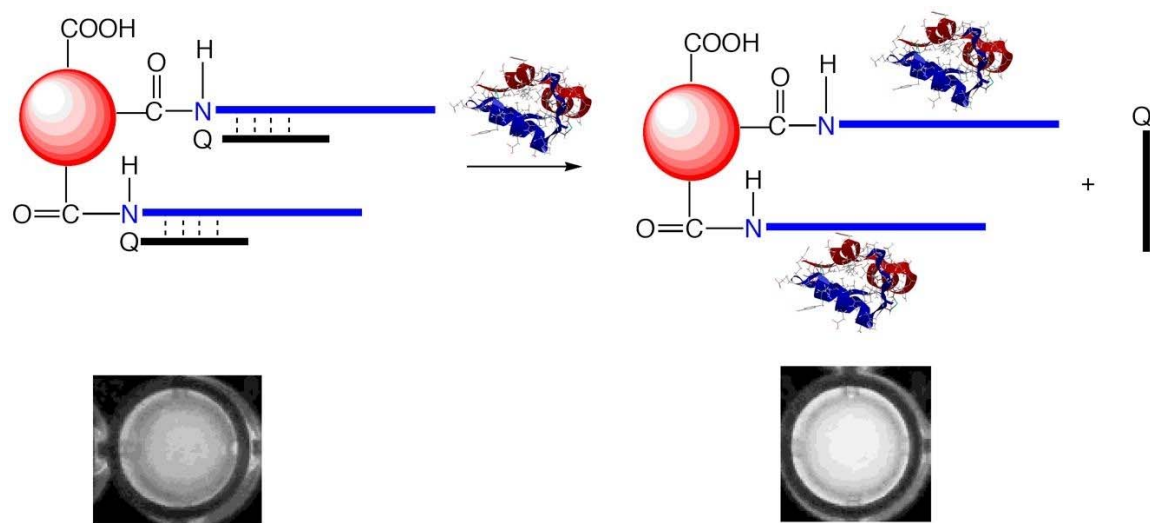
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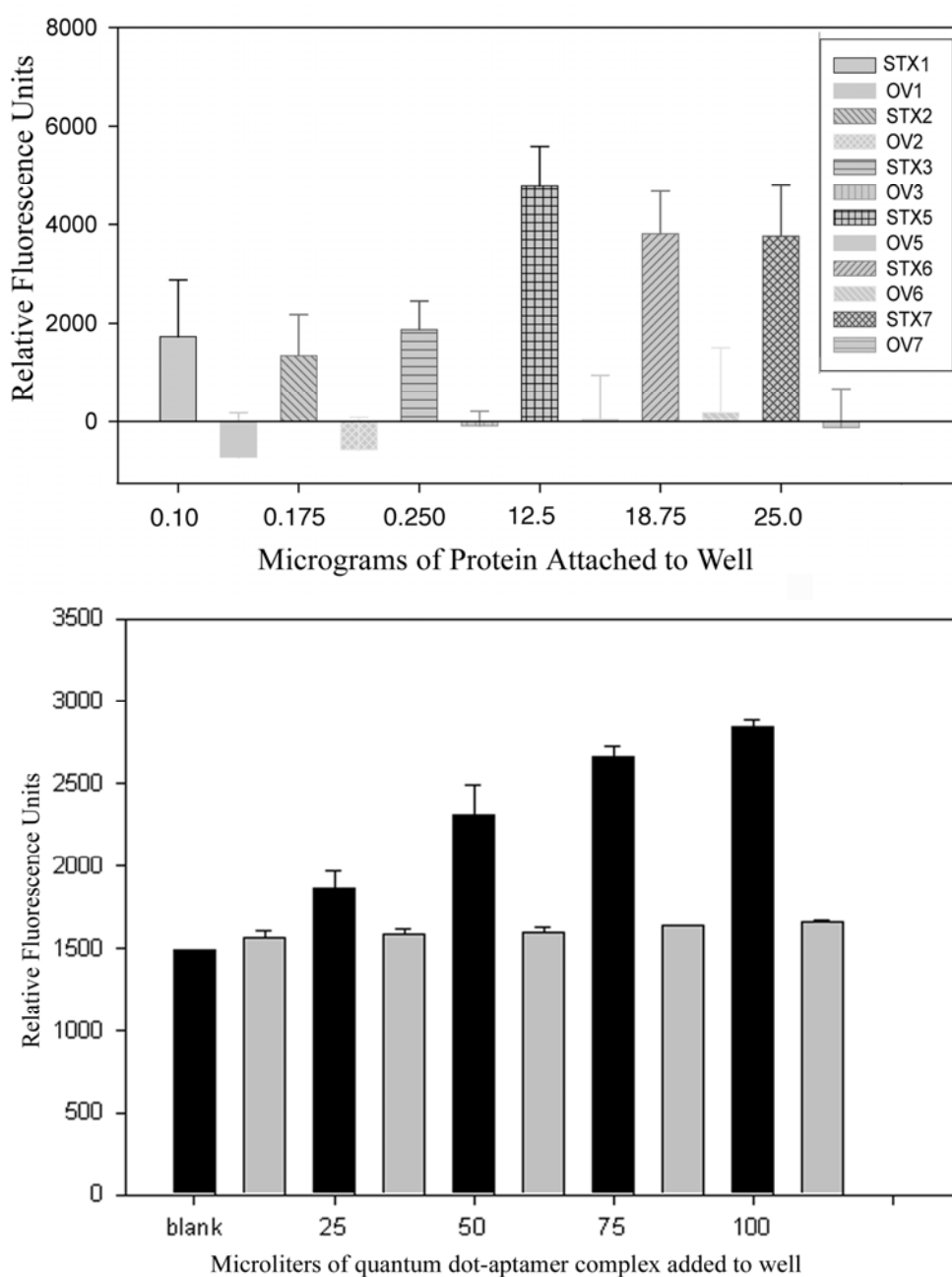
## KEY WORDS

Aptamers, tularemia, Shiga toxin, anthrax spores, bioterrorism, identification, quantum dots

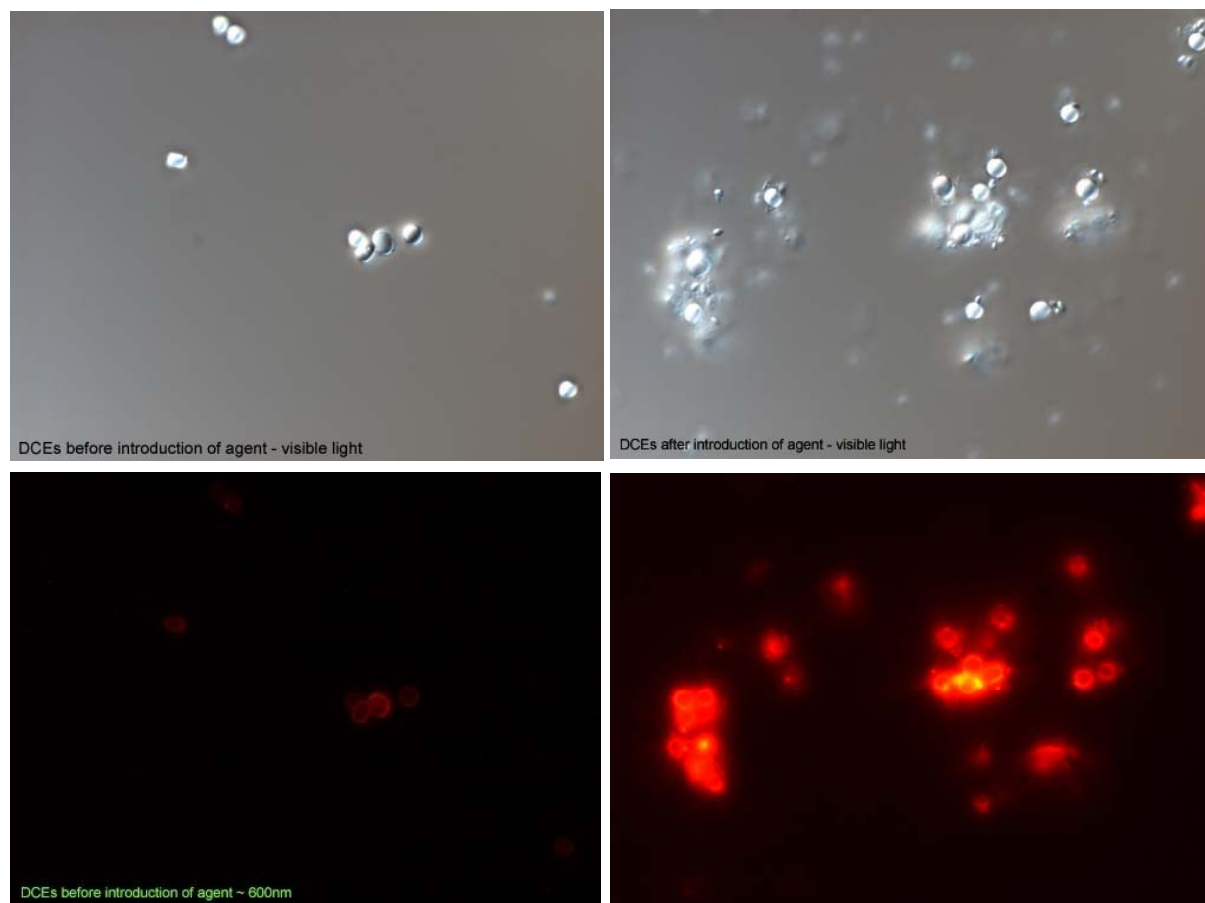
## FIGURES



**Figure 1.** Diagram of fluorescent quencher and nanocrystal (quantum dot) each attached to a complementary strand, respectively, of double-stranded DNA. When the target (depicted as a molecular model) displaces the quencher strand from the quantum dot strand, the complex is dequenched. The two wells shown below the diagram depict the two states of the complex: quenched (left) and dequenched (right).



**Figure 2.** Results of two formats of aptamer-linked immobilized sorbent assays (ALISA). On the top, the quenched quantum dot aptamer complex was immobilized in the wells of a microtiter plate and the specific ligand (Shiga toxin; STX) or a control (ovalbumin: OV) was added to the well at the quantities indicated. On the bottom, the Shiga toxin (black bars) or ovalbumin control (gray bars) was immobilized in each well of the microtiter plate and quantum dot suspension was added in the volumes indicated. The error bars represent standard errors of the mean.



**Figure 3.** Photomicrographs quenched aptamer/ quantum dot complex for anthrax spores (Sterne strain) immobilized on magnetic polybeads. Visible and UV lighted micrographs of quenched (no spores) beads on the left, and visible and UV lighted micrographs of dequenched beads on the right (with spores visible).



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